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Activation of Adenosine A_{2A} Receptors Inhibits Neutrophil Transuroepithelial Migration[▽]

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Adenosine has been identified as a significant inhibitor of inflammation by acting on adenosine A_{2A} receptors. In this study, we examined the role of adenosine and A_{2A} receptors in the transmigration of human neutrophils across an *in vitro* model of the transitional bladder urothelium. Human uroepithelial cells (UROtsa) were grown on transwell inserts; uropathogenic *Escherichia coli* (UPEC) and neutrophils were added to the transwell system; and the number of migrating neutrophils was evaluated. Reverse transcription-PCR (RT-PCR), immunohistochemistry, and flow cytometry were used to investigate the expression of adenosine receptors, the epithelial adhesion molecule ICAM-1, and the neutrophil integrin CD11b. Levels of proinflammatory interleukin-8 (IL-8) and phosphorylated IκBα were measured by enzyme-linked immunosorbent assays (ELISA) and Luminex assays, respectively. The neutrophils expressed all four adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3 receptors), but A_3 receptors were not expressed by UROtsa cells. UPEC stimulated neutrophil transuroepithelial migration, which was significantly decreased in response to the specific A_{2A} receptor agonist CGS 21680. The inhibitory effect of CGS 21680 on neutrophil migration was reversed by the A_{2A} receptor antagonist SCH 58261. The production of chemotactic IL-8 and the expression of the adhesion molecule ICAM-1 or CD11b were not significantly affected by CGS 21680. However, a significant decrease in the level of phosporylated IκBα was revealed in response to CGS 21680. In conclusion, UPEC infection *in vitro* evoked neutrophil migration through a multilayered human uroepithelium. The UPEC-evoked neutrophil transmigration decreased in response to A_{2A} receptor activation, possibly through inhibition of NF-κB signaling pathways.

Epithelial cells lining the urinary tract not only function as a mucosal barrier but also play an active role in host defense and constitute the first line of defense against uropathogenic bacteria, such as uropathogenic *Escherichia coli* (UPEC). The uroepithelial cells are activated by uropathogenic bacteria to secrete chemokines and cytokines, such as interleukin-8 (IL-8) and IL-6, and as a result, inflammatory cells are recruited into the tissue (27). Neutrophils are able to detect and migrate toward concentration gradients of chemotactic substances released by affected tissues or bacterial pathogens. During a urinary tract infection (UTI), neutrophils are the predominant inflammatory cells and are recruited to the site of infection in response to IL-8 (15). Several *in vitro* studies have investigated neutrophil migration through transwell inserts using nonpolarized single layers of urinary tract epithelial cells (2, 15). Neutrophil adhesion to urinary epithelium is mediated by CD11b/CD18 expressed on neutrophils (2, 23) and ICAM-1 (an adhesive receptor for the CD11b/CD18 integrin) expressed on uroepithelial cells (2). Neutrophils are an essential part of the innate immune system, and recruitment of these immune cells to infected tissues is crucial during the immune response. However, aberrant activation of neutrophils may also cause damage to normal tissue, for example, by excess release of reactive oxygen species (18, 30). It was recently shown that IL-8 receptor knockout mice have a defective neutrophil migration response, which resulted in neutrophil accumulation and bacteremia in an experimental UTI model (13).

The nucleotide ATP is known to be released from cells under stress conditions, and extracellular ATP can be metabolized to adenosine by ectonucleotidases, such as CD39 and CD73 (33). Adenosine activates four known adenosine receptor subtypes, the A_1, A_{2A}, A_{2B}, and A_3 receptors (12), of which the A_1, A_{2A}, and A_{2B} receptors are expressed in human urinary tract epithelial cells (25). Adenosine has been identified as a significant inhibitor of inflammation and cell damage (17), but the role and significance of adenosine during UTI are not known. One mechanism by which adenosine may affect inflammation is the regulation of neutrophil function. Adenosine has been shown to reduce neutrophil cytotoxic function, including adhesion, oxygen radical production, and production of tumor necrosis factor alpha (TNF-α) (10, 31). Interestingly, adenosine does not appear to inhibit all functions of neutrophils to the same extent. While extracellular superoxide release was strongly suppressed, phagocytosis was only moderately inhibited (31). Neutrophil chemotaxis can be modulated by extracellular ATP and adenosine (6, 11), and recent studies have shown that ATP is released and adenosine formed at the leading edge of migrating neutrophils to promote cell migration (5). Stimulation of $P2Y_2$ receptors and adenosine A_3 receptors has been shown to amplify chemoattractant-induced neutrophil migration (5, 20). In contrast, activation of adenosine A_{2A} receptors decreases the tissue-damaging activity of neutrophils

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by inhibiting the production of cytokines, the generation of superoxide anions, and the expression of adhesion molecules (7, 19).

The aim of the present study was to evaluate the role of adenosine and A_{2A} receptor activation in neutrophil migration across a polarized multilayer of human uroepithelial cells that resembles the transitional bladder urothelium.

MATERIALS AND METHODS

Human urinary tract epithelial cells. The human uroepithelial cell line UROtsa was kindly provided by Scott Garrett (University of North Dakota). UROtsa cells are derived from the ureter and were immortalized by using simian virus 40 (SV40) large T antigen. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma-Aldrich) at 37°C under a humidified 5% CO_2 atmosphere and were subcultured when confluent.

Preparation of neutrophils. Neutrophils were freshly isolated from blood samples taken from human volunteers. Briefly, total leukocytes and red blood cells were isolated by sedimentation with 6% (wt/vol) dextran T-500 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The supernatant was layered onto Histopaque-1077 (Sigma-Aldrich) and was centrifuged at 370 × *g* for 30 min. The pellet was washed in phosphate-buffered saline (PBS), centrifuged at 320 × *g* for 5 min, and subsequently treated with a cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA [pH 7.4]) for 30 min. This procedure resulted in >90% neutrophils, as assessed by microscopic evaluation and by use of a Swelab AC920EO AutoCounter (Boule Medical AB, Stockholm, Sweden). The viability of the cells was >98%, as determined by trypan blue exclusion.

Bacteria. UPEC strain IA2 was originally isolated from a patient with acute pyelonephritis. IA2 was cultured on tryptic soy agar (TSA) plates (Becton, Dickinson and Company, Sparks, MD) at 37°C.

Preparation of transwell UROtsa cell layers. UROtsa cells were seeded onto inverted 3-μm-pore-size polyethylene terephthalate (PET) transwell inserts (Falcon; BD Biosciences) and were allowed to settle for 3 h at 37°C under a 5% CO_2 atmosphere. The transwell inserts were then placed in 6-well plates (Falcon; BD Biosciences) with fresh medium and were incubated at 37°C under a 5% CO_2 atmosphere. When confluent, the cells were cultured in serum-free DMEM supplemented with 10 ng/ml epidermal growth factor (EGF), 4 pg/ml 3,3′,5-triiodo-L-thyronine, 12 ng/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite (all from Sigma-Aldrich) for an additional 2 weeks in order to obtain a polarized cell layer (24).

Neutrophil transuroepithelial migration. Serum-free culture medium (without penicillin and streptomycin) containing 10^8 CFU/ml of UPEC strain IA2 was added to the bottom well of transwell inserts for 24 h. Bacterial multiplication was limited by incubating the UROtsa cells with gentamicin (50 μg/ml) 24 h prior to infection. Gentamicin was excluded during infections. Human neutrophils were prepared as described above, and 3×10^6 neutrophils were added to the top well. Samples were taken from the bottom well after 0, 1, 2, and 3 h. The number of neutrophils that had migrated was counted in a Bürker chamber. To examine the role of adenosine receptor activation in transuroepithelial neutrophil migration, adenosine (10 μM) (Sigma-Aldrich) or the specific A_{2A} receptor agonist CGS 21680 [2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine] (1 μM) (Sigma-Aldrich) was added to the top well together with neutrophils after 24 h of UPEC infection. To confirm the involvement of A_{2A} receptors, the A_{2A} receptor antagonist SCH 58261 (10 nM; Sigma-Aldrich) was included in some experiments. To evaluate the cell-specific effects of CGS 21680, UROtsa cells or neutrophils were pretreated separately with CGS 21680 (1 μM) for 30 min before the start of the migration assay. To study the role of IL-8 in neutrophil transuroepithelial migration, a monoclonal anti-human CXCL8/IL-8 antibody (R&D Systems, Minneapolis, MN) was added to the top and bottom wells with neutrophils after 24 h of UPEC infection.

Preparation of UROtsa cells on inserts for light microscopy evaluation. Transwell inserts with multilayered UROtsa cells were washed in PBS and were fixed with cold 4% formaldehyde for 2 h. Cells were subsequently washed with sodium cacodylate buffer and were postfixed in 1% osmium tetroxide in cacodylate buffer for 40 min. Cells were dehydrated in a graded series of ethanol, followed by polymerization in Epon (Agar 100) at 60°C for 48 h. Sections (thickness, 2 to 4 μm) were cut from the membranes and stained with Azure II/methylene blue for light microscopic analysis.

Immunocytochemistry. UROtsa cells grown on transwell inserts were washed in PBS and fixated with cold 4% formaldehyde for 2 h. The membrane was placed in PBS with 0.2% bovine serum albumin (Sigma-Aldrich) and 10% goat serum (Serotec, Oxford, United Kingdom) for 30 min to block unspecific binding sites. Cells were incubated overnight at 4°C with a mouse monoclonal antibody against the human A_{2A} receptor (Santa Cruz) diluted 1:50, followed by an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody diluted 1:200 together with Alexa Fluor 546-phalloidin diluted 1:200 (both from Molecular Probes, Eugene, OR) for actin visualization. Control experiments to check the specificity of the anti-A_{2A} antibody were performed by omitting the primary antibody and adding only the secondary antibody. Fluorescence was examined using a Nikon Eclipse E600 confocal microscope (Nikon, Japan) and Nikon confocal microscope EZ-C1 2.20 software.

IL-8 ELISA analysis. The cell culture medium from the top and bottom wells in the neutrophil migration study was analyzed for IL-8 levels after 3 h. The medium was centrifuged at 5,000 × *g* for 5 min, and IL-8 was analyzed with the BD OptEIA human IL-8 ELISA (enzyme-linked immunosorbent assay) kit II (BD Biosciences Pharmingen, San Diego, CA). IL-8 was determined by measuring optical density at 450 nm with a Labsystems Multiskan Plus fluorescence spectrophotometer.

Preparation of total RNA and RT-PCR. RNA was isolated from UROtsa cells and neutrophils by using an RNeasy Mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Preparation of total RNA from neutrophils required DNase treatment (Qiagen Inc.). Total RNA from UROtsa cells (1 μg) and neutrophils (0.5 μg) was converted to cDNA using oligo(dT_{16}) primers (1 μM) (Applied Biosystems, Foster City, CA) and the Omniscript RT (reverse transcription) kit (Qiagen Inc.). RNA was controlled for genomic DNA contamination. PCR (reaction volume, 25 μl) was performed using Ready-To-Go PCR beads (Amersham Biosciences, Buckinghamshire, United Kingdom). Two microliters of the RT reaction product was mixed with primers (0.5 μM) for the A_1, A_{2A}, A_{2B}, or A_3 receptor or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1), and PCR was performed in a thermal cycler (Perkin-Elmer GeneAmp PCR system). PCR was run with initial denaturation for 1 min at 94°C, followed by 35 cycles at 94°C for 1 min, 65°C for 30 s, and 1 min at 72°C, and a final extension at 72°C for 10 min for the A_1 and A_{2A} receptors. For the A_{2B} and A_3 receptors, the initial denaturation was run for 4 min at 94°C, followed by 35 cycles at 94°C for 45 s, 60°C for 45 s, and 2 min at 72°C, and a final extension at 72°C for 7 min. PCR products were analyzed by agarose gel (2%) electrophoresis and were visualized by ethidium bromide staining.

Flow cytometric analysis of ICAM-1 and CD11b expression. UROtsa cells were stimulated with IA2 (10^6 CFU/ml) for 24 h as described above. After 24 h of stimulation, the cells were washed with PBS and were incubated with or without 1 μM CGS 21680 for 3 h. The cells were washed and detached from the surface by incubation with a 0.25% trypsin-EDTA solution (Gibco Invitrogen). The cells (1.5×10^5) were resuspended in PBS and were incubated either with a fluorescein isothiocyanate (FITC)-conjugated purified mouse monoclonal antibody against human CD54 (ICAM-1) (Beckman Coulter Inc., CA) or with an FITC-conjugated purified mouse monoclonal IgG1 antibody (Beckman Coulter Inc.) for 30 min in the dark at 4°C. Neutrophils were freshly isolated from peripheral blood obtained from healthy human volunteers. Briefly, the EDTA-treated blood was run on a Polymorphprep density gradient (Axis-Shield PoC AS, Oslo, Norway) according to the manufacturer's instructions. Osmolarity was restored by the addition of 0.45% NaCl. Erythrocytes were removed from the neutrophil suspension by hypotonic lysis. The resulting cell suspension contained >95% neutrophils as evaluated by microscopic examination. Neutrophils suspended in PBS either were not pretreated or were pretreated with 1 μM CGS 21680 for 2 h, followed by stimulation with 0.5 μg/ml lipopolysaccharide (LPS) (*E. coli* serotype 0127:B8; Sigma) for 3 h. Neutrophils (3×10^6) were resuspended in PBS and were incubated either with an R-phycoerythrin (RPE)-conjugated mouse monoclonal antibody against human CD11b (also known as the C3bi receptor) (Beckman Coulter Inc.) or with Tri-Color-conjugated mouse monoclonal IgG1 (Beckman Coulter Inc.) for 30 min in the dark at 4°C. The cells were washed once with PBS, fixed with 0.1% formaldehyde, and analyzed by flow cytometry (Coulter Epics Altra; Beckman Coulter Inc.). The mean ratios of fluorescence intensity for ICAM-1, CD11b, and IgG1 were calculated and compared.

Analysis of phosphorylated IκBα by Luminex assay. UROtsa cells were seeded in 24-well plates, and when they were confluent, normal medium was replaced with serum-free medium and cells were incubated for 4 h. Thereafter, UROtsa cells were stimulated with CGS 21680 (1 μM) for 7 or 15 min. UROtsa cells stimulated with UPEC strain IA2 (10^8 CFU/ml) for 30 min and a HeLa cell extract stimulated with TNF-α (100 ng/ml) for 7 min were used as positive

TABLE 1. PCR primer sequences

Primer	Orientation	Sequence	Product size (bp)
A_1R	Forward	5′-CTA CCT AAT CCG CAA GCA GC-3′	367
	Reverse	5′-GTC ATC AGG CCT CTC TTC TGG-3′	
$A_{2A}R$	Forward	5′-AAC CTG CAG AAC GTC ACC A-3′	245
	Reverse	5′-GTC ACC AAG CCA TTG TAC CG-3′	
$A_{2B}R$	Forward	5′-GTG CCA CCA ACA ACT GCA CAG AAC-3′	517
	Reverse	5′-CTG ACC ATT CCC ACT CTT GAC ATC-3′	
A_3R	Forward	5′-CAC CAC CTT CTA TTT CAT TGT CTC T-3′	337
	Reverse	5′-GGT ACT CTG AGG TCA GTT TCA TGT T-3′	
GAPDH	Forward	5′-ATT CCA TGG CAC CGT CAA GGCT-3′	571
	Reverse	5′-TCA GGT CCA CCA CTG ACA CGT T-3′	

controls. Cells were lysed with lysis buffer (provided by the Luminex kit) supplemented with Complete Mini, a protease inhibitor cocktail (Roche Diagnostics, Pleasanton, CA), on an orbital shaker. The lysate was filtered in Spin-X centrifugation tubes (0.22-μm-pore-size filter) (Sigma) at 14,000 × g for 1 min and was then stored at −70°C until analysis. Unstimulated cells were used as controls. The protein concentrations of the samples were determined with the bicinchoninic acid (BCA) protein assay kit (Pierce). Equal amounts of protein (5 μg) were analyzed for IκBα with the Milliplex Map multipathway signaling kit (Millipore Corp., Billerica, MA) according to the manufacturer's instructions on a Luminex 200 instrument (Millipore). Data are expressed as the mean fluorescence intensity (MFI).

Statistical analysis. Data are shown as means ± standard errors of the means (SEM), and *n* indicates the number of independent experiments. Student's unpaired *t* test or one-sample *t* test analyses were used when two treatments were compared. For multiple comparisons, analysis of variance (ANOVA), followed by Bonferroni's or Dunnett's posttest, was used. *P* values of <0.05 were considered statistically significant.

RESULTS

Establishment of a polarized multilayered uroepithelium. Light microscopy studies of cross-sectioned UROtsa cells on transwell insert membranes confirmed that UROtsa cells form a multilayered polarized uroepithelium when cultured in serum-free medium (Fig. 1A and B). Approximately 3 to 4 cell layers were formed, and the cells within the basal cell layer appeared to be smaller than the cells in the upper cell layer.

Multilayered UROtsa cells on transwell inserts were stimulated by the addition of the UPEC strain IA2 to the bottom well. After 24 h, neutrophils were added to the top well, and the number of neutrophils that had migrated was determined in the bottom well after 0, 1, 2, and 3 h. After 2 h, neutrophil migration reached a plateau, and the number of migrating neutrophils was approximately 6-fold higher across cell layers exposed to UPEC than across unstimulated cell layers (Fig. 1C).

Expression of adenosine receptors. RT-PCRs were performed to study the basal mRNA expression of adenosine receptors in UROtsa cells and neutrophils. As shown in Fig. 2A, transcripts for the A_1 (367 bp), A_{2A} (245 bp), and A_{2B} (517 bp) receptor subtypes, but not for the A_3 (337 bp) receptor subtype, were detected in UROtsa cells. The strongest expression was found for the A_{2B} transcript, while A_1 and A_{2A} expression was weaker. Transcripts for all four receptor subtypes were observed in neutrophils, in which the expression of the A_{2A} and A_3 transcripts was clearly stronger than that of the A_1 and A_{2B} transcripts (Fig. 2B). Protein expression of the A_{2A} receptor subtype was confirmed by immunocytochemistry of UROtsa cells grown on transwell inserts (Fig. 2C). In control experiments, no A_{2A} immunoreactivity was detected in sections incubated with the secondary antibody only (Fig. 2D).

Effect of adenosine receptor activation on neutrophil transuroepithelial migration. In order to examine the effect of adenosine receptor activation on UPEC (IA2)-evoked neutrophil transuroepithelial migration, adenosine (10 μM; $n = 6$) or the specific A_{2A} receptor agonist CGS 21680 (1 μM) was added to the top well. The migration of neutrophils was not altered in response to adenosine (Fig. 3A). However, when cells were stimulated with the A_{2A} receptor agonist CGS 21680, UPEC-evoked migration was significantly decreased after 1 h ($n = 8$; $P < 0.01$) and 2 h ($n = 7$; $P < 0.001$) (Fig. 3B). The inhibitory effect of CGS 21680 on migration was abolished in the presence of the A_{2A} receptor antagonist SCH 58261 (10 nM; $n = 2$), confirming the involvement of the A_{2A} receptor

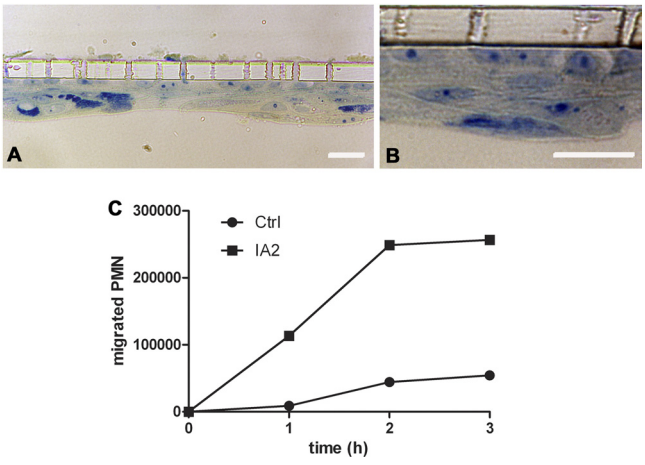

FIG. 1. Light microscopy showing cross-sectioned UROtsa cell layers on transwell insert membranes. (A and B) UROtsa cells cultured in serum-free medium for 14 days form a multilayered, polarized uroepithelium. Bars, 20 μm. (C) Representative graph showing the migration of neutrophils through unstimulated (Ctrl) (●) or UPEC (IA2)-stimulated (■) UROtsa cell layers over time.

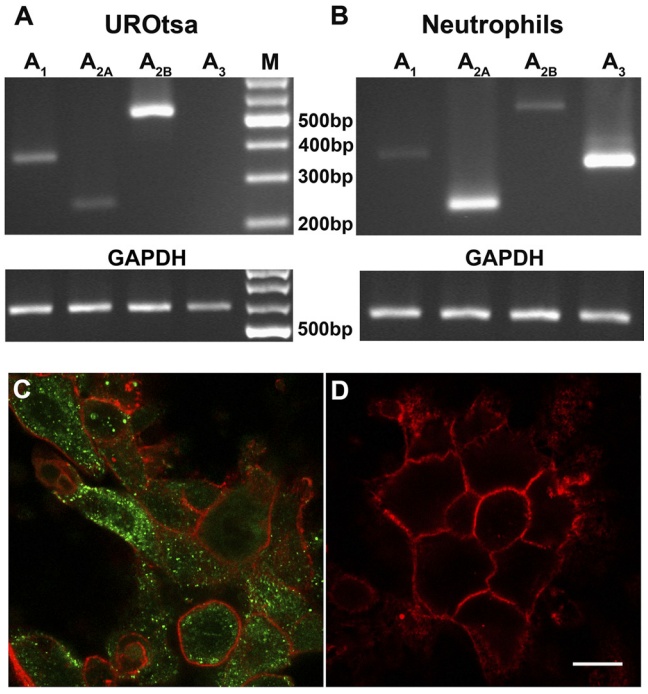

FIG. 2. (A and B) RT-PCR of the A_1 (367 bp), A_{2A} (245 bp), A_{2B} (517 bp), and A_3 (337 bp) receptor subtypes and GAPDH (571 bp) in UROtsa cells (A) and neutrophils (B). (C) Immunocytochemical labeling of A_{2A} receptors in polarized UROtsa cells. Actin filaments (red) were stained with Alexa Fluor 546-phalloidin, and the A_{2A} receptor was visualized with an Alexa Fluor 488-conjugated secondary antibody (green). (D) Control experiment in which the A_{2A} antibody has been omitted and only the secondary antibody has been added. Actin filament staining with phalloidin is shown for general visualization of the cells. Bar, 20 μm.

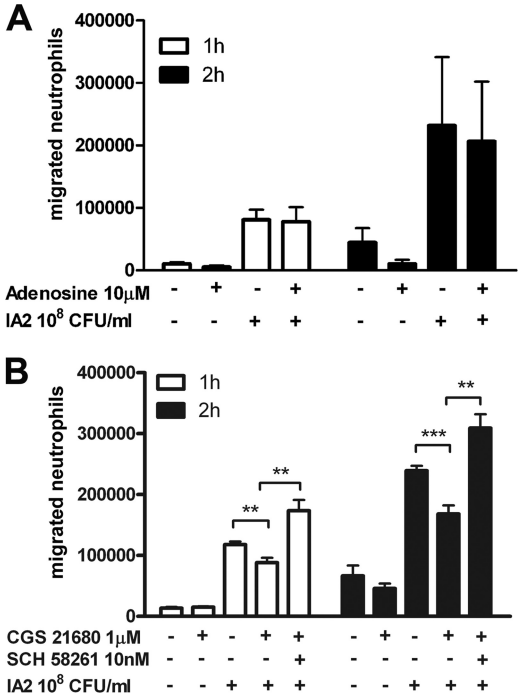

FIG. 3. Activation of adenosine receptors and neutrophil transuroepithelial migration. Neutrophil migration across unstimulated or UPEC (IA2)-stimulated UROtsa cell layers was studied in the presence of adenosine (10 μM) (A) or the specific A_{2A} receptor agonist CGS 21680 (1 μM) in the absence or presence of the A_{2A} receptor antagonist SCH 58261 (10 nM) (B). Results are expressed as means ± SEM. Asterisks indicate statistical significance (**, $P < 0.01$; ***, $P < 0.001$).

subtype (Fig. 3B). In an attempt to find out whether the observed effects of CGS 21680 were caused by an effect on neutrophils or on UROtsa cells, each cell type was pretreated separately with CGS 21680 before the start of the migration assay. However, migration was not significantly inhibited when only the neutrophils or only the UROtsa cells were pretreated with CGS 21680 (data not shown).

Role of IL-8 in neutrophil transuroepithelial migration. To confirm the role of IL-8 in neutrophil transuroepithelial migration (14), a monoclonal anti-IL-8 antibody (1 μg/ml [$n = 4$] and 10 μg/ml [$n = 3$]) was added to the medium. UPEC (IA2)-induced neutrophil migration was significantly reduced after 1 and 2 h by the addition of the anti-IL-8 antibody (Fig. 4A). We also measured IL-8 levels in the top and bottom wells of the transwell system to find out if CGS 21680 affects migration by reducing IL-8 production. Increased IL-8 levels were detected in the medium from both the top well ($n = 5$; $P < 0.01$) and the bottom well ($n = 5$; $P < 0.001$) of UPEC-exposed cells analyzed after 3 h (Fig. 4B). Separate studies showed that most of the IL-8 produced was derived from neutrophils (data not shown). Thus, the high IL-8 levels in the top well are likely caused by the presence of nonmigrating neutrophils in this well. The A_{2A} receptor agonist CGS 21680 ($n = 5$) did not affect UPEC-induced IL-8 production either in the top well or in the bottom well (Fig. 4B). These data suggest that the reduced migration in response to CGS 21680 is not associated with changes in IL-8 levels.

Effect of CGS 21680 on ICAM-1 and CD11b expression. The adhesion molecule ICAM-1 on uroepithelial cells and the neutrophil integrin CD11b are known to play active roles in neutrophil migration during urinary tract infections (2). We next examined whether the decreased neutrophil migration evoked by CGS 21680 was associated with changes in ICAM-1 or CD11b expression. Stimulation of UROtsa cells with UPEC (IA2) increased ICAM-1 expression over that by unstimulated control cells (data not shown). IA2-stimulated UROtsa cells were incubated with CGS 21680 and were analyzed for ICAM-1 expression by flow cytometry. The mean percentage of ICAM-1 fluorescence intensity following stimulation with CGS 21680 was not different (98% ± 8.1%; $n = 6$) from that of cells without CGS 21680 stimulation (MFI set to 100%) (Fig. 5A). Stimulation of neutrophils with LPS increased CD11b expression over that by unstimulated control cells (data not shown). To test whether CGS 21680 affected the expression of CD11b, neutrophils were treated with CGS 21680 and LPS and were analyzed by flow cytometry. The mean percentage of CD11b fluorescence intensity increased slightly, but not significantly, following stimulation with CGS 21680 (112% ± 12%; $n = 4$) over that for cells without CGS 21680 stimulation (set to 100%) (Fig. 5B). Taken together, these data suggest that neither ICAM-1 nor CD11b expression is affected by CGS 21680.

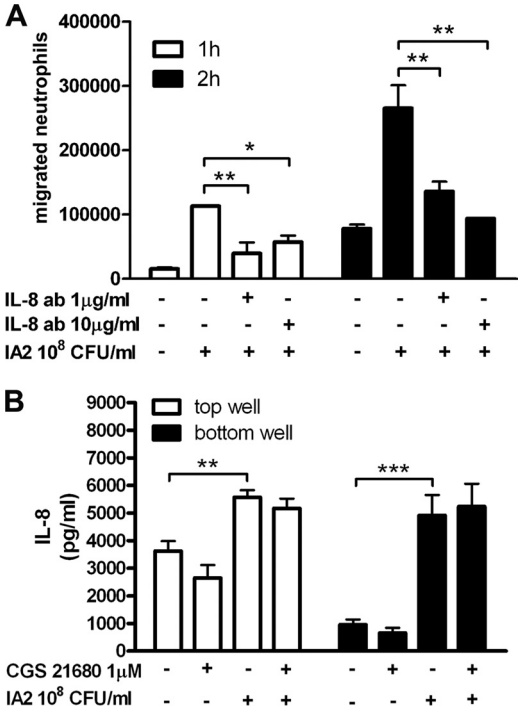

FIG. 4. Role of IL-8 in neutrophil transuroepithelial migration. (A) UPEC (IA2)-evoked neutrophil transuroepithelial migration in the presence of a monoclonal anti-IL-8 antibody (ab). Asterisks indicate statistically significant differences from IA2-treated cells (*, $P < 0.05$; **, $P < 0.01$). (B) IL-8 levels were measured in the top and bottom wells of IA2-infected UROtsa cells and neutrophils after 3 h in the absence or presence of the A_{2A} receptor agonist CGS 21680 (1 μM). Results are expressed as means ± SEM. Asterisks indicate statistically significant differences from unstimulated cells (**, $P < 0.01$; ***, $P < 0.001$).

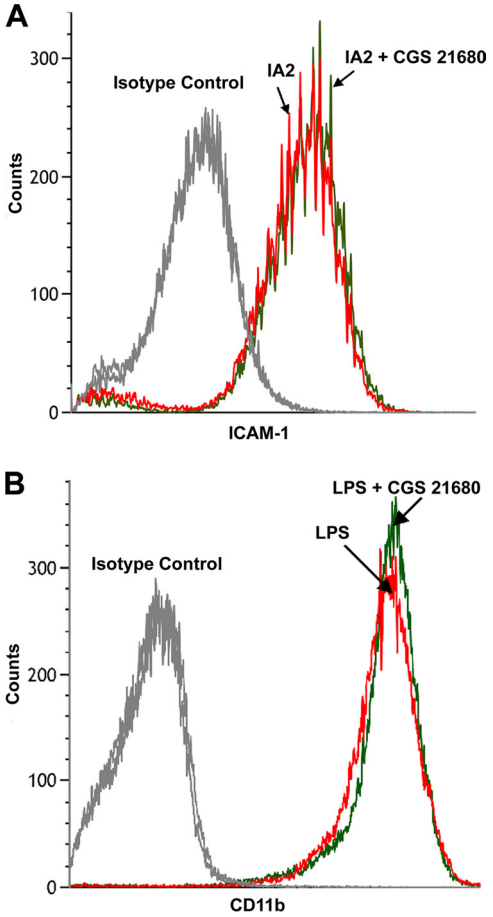

FIG. 5. (A) ICAM-1 expression in UPEC (IA2)-stimulated UROtsa cells after stimulation with CGS 21680 (1 μM). Cells were stained either with an antibody against CD54 (ICAM-1) or with the isotype control IgG1 and were analyzed by flow cytometry. (B) CD11b expression in LPS-stimulated neutrophils after stimulation with CGS 21680 (1 μM). Neutrophils were stained either with anti-CD11b or with the isotype control IgG1 and were analyzed by flow cytometry. Histograms representative of four (CD11b) and six (ICAM-1) separate experiments are shown.

Effect of CGS 21680 on phosphorylated IκBα. The exposure time of CGS 21680 in the transmigration assay is short (2 to 3 h), and in order to be able to detect early anti-inflammatory changes, we measured the levels of phosphorylated cytosolic IκBα. CGS 21680 (1 μM) reduced the mean fluorescence intensity of phosphorylated IκBα from 21 ± 4.3 to 10 ± 0.65 after 7 min ($n = 4$; $P < 0.05$) and from 25 ± 4.7 to 16 ± 2.9 after 15 min (Fig. 6A). UPEC (IA2; 10^8 CFU/ml)-stimulated UROtsa cells ($n = 4$) and TNF-α-stimulated HeLa cells ($n = 3$) were used as positive controls; as expected, these cells showed increased levels of phosphorylated IκBα (Fig. 6B). These data suggest that CGS 21680 may inhibit NF-κB signaling pathways.

DISCUSSION

Many previous studies of neutrophil transuroepithelial migration have been performed using the A498 kidney (2, 15) and J82 bladder (2) epithelial cells, but these cells form a nonpolarized monolayer. The use of multilayered uroepithelial cells for migration studies better mimics the real scenario of UTI, where neutrophils are migrating across several layers of cells to reach the bladder lumen. UROtsa cells have previously been reported to form polarized multilayers (24), and we confirmed that these cells were able to form three to four layers when cultured under serum-free conditions on transwell inserts. It was shown that UPEC strain IA2 induced a consistent increase in the number of migrating neutrophils during the first 2 h, but then the migration reached a plateau. Neutrophil migration was minor across cell layers that had not been exposed to UPEC. The chemokine IL-8 is produced by urinary tract epithelial cells and neutrophils during inflammation (1, 21) and is known to be involved in neutrophil transuroepithelial migration (14). We have previously shown that UROtsa cells grown in a monolayer produce increased IL-8 levels when stimulated for 24 h with UPEC strain IA2 (26). In the present study, increased IL-8 production in response to UPEC infection was confirmed by greater accumulation of IL-8 in the bottom well of the transwell system for infected cells than for uninfected cells. In addition, monoclonal antibodies against IL-8 almost completely blocked UPEC-induced neutrophil migration, suggesting that IL-8 has a major role as a chemoattractant in transuroepithelial migration. These results are in agreement with those of a similar study conducted on monolayers of

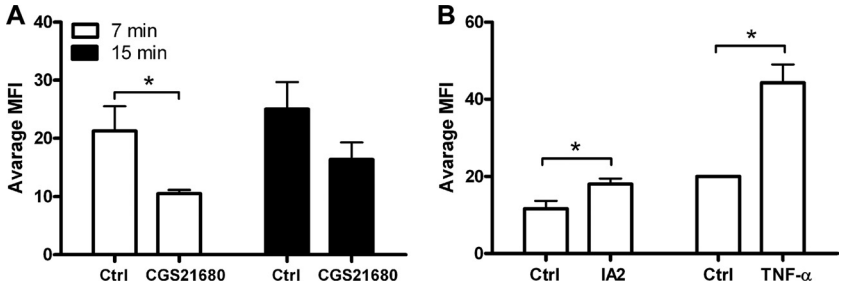

FIG. 6. (A) Analysis of phosphorylated IκBα after stimulation of UROtsa cells with CGS 21680 (1 μM) for 7 or 15 min ($n = 4$). (B) UROtsa cells stimulated with UPEC strain IA2 (10^8 CFU/ml) for 30 min ($n = 4$) or HeLa cell extracts stimulated with TNF-α (100 ng/ml) ($n = 3$) for 7 min were used as positive controls. The results are expressed as mean fluorescence intensity (MFI) and are given as means ± SEM. Asterisks indicate statistically significant differences from nonstimulated (Ctrl) cells (*, $P < 0.05$).

urinary tract epithelial cells infected with the *E. coli* strain Hu734 (15). The high levels of IL-8 noted in the top well after 3 h are likely caused by the accumulated basal release of IL-8 from the many nonmigrating neutrophils in the top well at this time point. Thus, after 3 h, the IL-8 gradient, promoting unidirectional migration of neutrophils, is no longer present. The time course study for the migration also reveals that the migration shows a plateau after 2 to 3 h, indicating that the IL-8 gradient has ceased.

Several studies performed on neutrophils have demonstrated that the production of cytokines and free radicals, as well as the expression of adhesion molecules, is decreased by activation of the adenosine A_{2A} receptor (7, 19, 31). Stimulation of neutrophils and UROtsa cells with the specific A_{2A} receptor agonist CGS 21680 reduced the migration of neutrophils across the UROtsa cell layer after both 1 and 2 h. The reduced migration evoked by CGS 21680 was attenuated by an A_{2A} receptor antagonist, suggesting that the inhibition was due to activation of the A_{2A} receptor. Additional experiments, in which the neutrophils and the UROtsa cells were pretreated separately with CGS 21680 before the start of the migration assay, were performed to find out if CGS 21680 affected mainly neutrophils or UROtsa cells. However, significant inhibition of neutrophil migration was seen only when neutrophils and UROtsa cells were coincubated with CGS 21680, suggesting that the reduced CGS 21680-mediated migration involves A_{2A} receptors both on neutrophils and on uroepithelial cells. RT-PCR data showed that the A_{2A} receptor is expressed at high levels in neutrophils, whereas A_{2A} receptor transcript levels were low in UROtsa cells. However, A_{2A} receptor protein was demonstrated in UROtsa cells by immunocytochemistry. Although the specific A_{2A} receptor agonist CGS 21680 reduced migration, adenosine was not able to affect UPEC-evoked neutrophil migration. A likely explanation is that adenosine activates adenosine receptors other than the A_{2A} receptor subtype—for example, the high-affinity A_1 subtype. Activation of both A_1 and A_3 receptors on neutrophils has been shown to mediate and amplify neutrophil migration (5, 8, 20). Thus, an inhibitory effect of adenosine on A_{2A} receptors is likely to be masked by activation of the A_1 and A_3 receptors, which stimulates neutrophil migration.

Experiments were performed to investigate whether the reduced migration evoked by CGS 21680 involved changes in the levels of chemotactic IL-8. However, stimulation of the A_{2A} receptor by CGS 21680 was not able to modulate the IL-8 production evoked by UPEC. These data suggest that changes in IL-8 levels cannot explain the decreased migration evoked by CGS 21680. In agreement with our data, McColl et al. (21). showed that neither adenosine nor A_{2A} receptor activation is able to downregulate LPS-induced IL-8 expression and production in neutrophils. However, in contrast to our findings, A_{2A} receptor activation did not affect neutrophil movement, at least not toward *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), IL-8, or leukotriene B_4 (21).

To further elucidate the mechanism by which A_{2A} receptor activation reduces neutrophil migration, we examined whether the expression of the adhesion molecule ICAM-1 was altered upon exposure to CGS 21680. Stimulation of A_{2A} receptors has previously been shown to decrease ICAM-1 immunoreactivity in kidney peritubular capillaries and also to reduce neutrophil infiltration in kidney tissue (22). Our results showed that UPEC-evoked ICAM-1 expression was unaltered by CGS 21680. Besides ICAM-1, there are several additional candidate epithelial receptors for migrating neutrophils, such as the immunoglobulin superfamily (IgSF) members junctional adhesion molecules and CD47 (34). However, the knowledge of these molecules in urinary tract epithelium and of their regulation by adenosine is limited. ICAM-1 on uroepithelial cells binds to the CD11b/CD18 counterpart on neutrophils (2), and A_2 receptor activation has previously been shown to inhibit fMLP-induced upregulation of CD11b/CD18 expression in neutrophils (32). However, in that study, the nonselective A_2 agonist 5′-*N*-ethylcarboxamidoadenosine was used, and the involvement of the A_{2A} receptor is therefore uncertain. We studied the effect of A_{2A} receptor activation on LPS-induced CD11b expression in neutrophils, but decreased expression of CD11b could not be confirmed using the selective A_{2A} agonist CGS 21680. However, the expression of CD11b on the neutrophil surface does not necessarily reflect the functional capacity of neutrophils, since our experiments give no information on the affinity state or the signaling capacity of CD11b. Moreover, it is possible that changes in the expression of adhesion molecules are difficult to detect within the short 3-h study period used in this study.

Another mechanism known to reduce the adhesion and transmigration of neutrophils is inhibition of NF-κB signaling (29). In this study, we measured the levels of phosphorylated cytosolic IκBα protein in UROtsa cells as an early marker for

possible changes in transmigration. Phosphorylation of the IκBα protein results in dissociation of IκBα from NF-κB, followed by translocation of free NF-κB into the nucleus (3). Stimulation of the A_{2A}-receptor by CGS 21680 significantly decreased IκBα phosphorylation, suggesting decreased activation of NF-κB signaling by CGS 21680 in UROtsa cells. The promoter of the ICAM-1 gene contains recognition sequences for NF-κB, and inhibition of IκBα phosphorylation has been shown to decrease ICAM-1 expression in TNF-α-stimulated human umbilical vein epithelial cells (HUVEC) (4). Taken together, our findings indicate that the reduced neutrophil migration in response to A_{2A} receptor activation may be associated with reduced NF-κB signaling.

Increased knowledge of endogenous mediators, such as adenosine, that regulate and limit neutrophil activation is of interest, since these are potential targets for inflammatory diseases associated with unchecked neutrophil activation. The significance of adenosine in the protection from neutrophil-derived cytotoxicity was reported to be substantial; inactivation of adenosine increased cytotoxicity toward endothelial cells by 40 to 70% (9). UPEC infection of IL-8 receptor knockout mice was recently shown to result in bacteremic pyelonephritis and renal scarring due to a dysfunctional neutrophil response (16, 28). Taken together, our results demonstrate that A_{2A} receptor activation regulates neutrophil migration across human uroepithelial cells *in vitro*. Further studies are needed to elucidate the significance of A_{2A} receptor signaling for neutrophil migration during UTI.

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